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Enzymically attaching oligosaccharide-linked ‘cargoes’ to cellulose and other commercial polysaccharides via stable covalent bonds

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Keywords

Cellulose modification, Hetero-transglycosylation, Xyloglucan

Abstract

~~Cellulose is a fundamental component of biomass and of many high technology materials. The~~
Equisetum enzyme hetero-trans- β -glucanase (HTG) covalently grafts native plant cellulose
(donor-substrate) to ~~(*celigo*)~~xyloglucans (acceptor-substrates), potentially offering a novel
'green' method of cellulose functionalisation. However, the range of cellulosic and non-
cellulosic donor substrates that can be utilised by current ignorance of HTG's donor-
substrate ~~HTG is unknown, range-limitings~~ our insight into its biotechnological potential. Here
we show that HTG ~~was able to bond~~ binds all celluloses tested (papers, tissues, hydrogels,
bacterial cellulose) to radioactively- or fluorescently-labelled xyloglucan-heptasaccharide
(XXXGol; acceptor-substrate). Glycol-chitin, glycol-chitosan and chitosan also acted as donor
substrates, but less effectively than cellulose, ~~donor-substrates~~. Cellulose-XXXGol conjugates
were formed throughout ~~a~~ the volume of a block of hydrogel's ~~volume~~, demonstrating
penetration. Plant-derived celluloses (cellulose I β) became more effective donor-substrates after
'mercerisation' in ≥ 3 M NaOH; the opposite was true for bacterial cellulose I α . Cellulose-
XXXGol bonds resisted boiling 6 M NaOH, demonstrating strong glycosidic bonding. In
conclusion, HTG stably grafts native and processed celluloses to xyloglucan-oligosaccharides,
which may carry valuable 'cargoes', exemplified by sulphorhodamine. We thus demonstrate
HTG's biotechnological potential to modify various cellulose-based substrates such as cellulosic
textiles, pulps, papers, packaging, sanitary products and hydrogels.

1. Introduction

Plant cell walls are complex polysaccharide composites, crucial for plant function and survival [1]. Wall polysaccharides fall into three classes: cellulose, hemicelluloses and pectin [2],[3]. Cellulose molecules typically comprise 1,000–10,000 (1→4)-linked β -D-glucose residues and these unbranched chains, synthesised at the plasma membrane, aggregate into microfibrils [4]. Pectin is a complex anionic polysaccharide composed of four major interlinked domains, which are rich in galacturonic acid residues [5]. Major land-plant hemicelluloses possess a β -(1→4)-linked backbone of β -D-glucose and/or β -D-mannose or β -D-xylose residues. Some hemicelluloses carry side chains: for example, xyloglucan, the most abundant hemicellulose in most land-plant primary cell walls [1], carries (1→6)-linked α -D-xylose side-chains, some of which themselves also bear β -D-Gal or α -L-Fuc-(1→2)- β -D-Gal attached to O-2. One plant hemicellulose — mixed-linkage β -glucan (MLG) — has ~25–30% β -(1→3)-bonds interspersed with the β -(1→4)-bonds of cellotriose and cellotetraose sequences. These three polysaccharide classes are considered to form dense networks [6].

Plant cell walls also contain numerous enzymes whose substrate specificity suggests that they act to restructure the wall's polysaccharides [7]. Here we focus on transglucanases which belong to glycoside hydrolase family 16 (GH16) [8],[9],[10]. The most intensively studied GH16s are xyloglucan endotransglucosylase/hydrolases (XTHs) [11],[12], which possess one or both of two activities: xyloglucan endotransglucosylase (XET; EC 2.4.1.207) and xyloglucan endohydrolase (XEH; EC 3.2.1.151). XET activity cuts a xyloglucan chain (donor substrate) and grafts it onto the non-reducing end of a neighbouring xyloglucan (or oligosaccharide thereof; the acceptor substrate), thus bringing about a xyloglucan:xyloglucan homo-transglucanase reaction [13],[14]. Land-plant genomes typically encode more than 30 XTHs [15]. Other examples of homo-transglycanase activities reported in plant extracts are trans- β -xylanase [16] and trans- β -mannanase [17].

In addition to enzymes that catalyse homo-transglycosylation reactions, recent studies report that certain transglucanases can cleave cellulose chains [18],[19], soluble cellulose derivatives [20],[21], or MLG, and subsequently attach them via glycosidic bonds to the xyloglucan acceptor substrate, creating hybrid products e.g. cellulose–xyloglucan or MLG–xyloglucan. Since the donor differs qualitatively from the acceptor, such reactions are termed hetero-transglycosylation; they have considerable biotechnological potential, giving a means of covalently attaching commercially significant polysaccharides (cellulose or MLG) to xyloglucan or its oligosaccharides. The latter can, in principle, carry valuable ‘cargoes’ which thereby become permanently attached to the cellulose or MLG.

In most reports of “cellulose” heterotransglucosylation, the substrate tested was an artificial, soluble, cellulose derivative (e.g. cellulose acetate, sulphocellulose, hydroxyethylcellulose or phosphoric acid-treated amorphous cellulose [19],[22]. In contrast, a heterotransglucanase that acts on unmodified cellulose, a major industrial raw material and the world’s most abundant organic substance, could have great commercial impact. The only hetero-transglucanase known to exhibit cellulose:xyloglucan endotransglucosylase (CXE) activity on native insoluble plant cellulose (cotton-sourced filter paper) is hetero-trans- β -glucanase (HTG), a highly acidic transglucanase discovered recently in the early-diverging ‘fern’ genus *Equisetum* [18]. All other tested acidic transglucanases from *Equisetum* are XTHs with very predominantly homo-transglucanase activity [23]. Besides CXE activity, *Ef*HTG catalyses two further transglucosylation reactions at high rates — MLG:xyloglucan endotransglucosylase (MXE) and ~~‘classical’~~ordinary XET activity [18],[24],[25].

The aim of the present study was to provide the first thorough investigation of the influence of the cellulose type and origin on hetero-transglucosylation by HTG. We also further explored the range of donor substrates tolerated by HTG, and we tested the chemical robustness of the cellulose–xyloglucan bonds formed by this unique enzyme.

2. Experimental

2.1. Plant sources and materials

Equisetum fluviatile was obtained from a pond outside the Institute of Molecular Plant Sciences at the University of Edinburgh or from the Pentland Hills (Edinburgh, UK). Polysaccharides purchased from Sigma-Aldrich were as follows: chitin (from shrimp shells; C7170), chitosan 1 (low M_r , 75–85% deacetylated; 448869), chitosan 2 (medium M_r , 75–85% deacetylated; 448877), chitosan 3 (high M_r , > 75% deacetylated; 419419) chitosan 4 (from crab shells, ~80% deacetylated; C0792), chitosan 5 (from shrimp shells, \geq 75% deacetylated; C3646), glycol chitosan (DP > 400, \geq 60% deacetylated; G7753), fucoidan (from *Fucus Vesiculosus*, F5631), alginate (from brown macroalgae; W201502), λ -carrageenan (from *Gigartina aciculare* and *G. pistillata*; C3889,) laminarin (M_r ~4500; L9634), pectin (from Citrus fruit, \geq 85% esterified; P9561), homogalacturonan (polygalacturonic acid, from citrus fruit, 85-90%; P7276), arabinogalactan (from larch wood; 10830), xylan (from birchwood, M_r 98,066, 92% soluble, 88% xylose, 10% hexuronic acids, < 10% arabinose; X0502), xylan (from oat spelts, ~90% xylose, < 10% arabinose; 95590), 4-*O*-methylglucuronoxylan (from *Fagus sylvatica*, M-5144; 96% soluble, 13% hexuronic acids), α -mannan (from *Saccharomyces cerevisiae*; M7504) and microcrystalline cellulose (M_r ~29,000; 11365). Polysaccharides from Megazyme were: wheat arabinoxylan (Ara:Xyl ratio 38:62, M_r 192,000; P-WAXYM), arabinan (from sugar beet pulp; Ara: Gal: Rha: GalA = 88: 3: 2: 7; P-ARAB), linear arabinan (debranched, Ara: GalA: Rha: GalA = 97.5:0.4:0.1:2, M_r 18,000; P-DBAR), arabinogalactan (from larch wood, Gal: Ara: Other sugars = 81: 14: 5, M_r 47,000; P-ARGAL), galactan (from potato, Gal: Ara: Rha: GalA = 87: 3: 4: 6; P-GALPOT), arabinoxylan medium viscosity (from wheat; M_r 323,000; P-WAXYM), lichenan (MLG from Iceland moss, 1,4:1,3- β -D linkage ratio 2:1; P-LICHN), MLG low viscosity (from barley, M_r 179,000, 1,4:1,3- β -D linkage ratio 3:1; P-BGBL), MLG medium viscosity (from barley, M_r 251,000; P-BGBM) and high viscosity (from barley, M_r 495,000; P-BGBM), rhamnogalacturonan I (from potato fiber; GalA: Rhamnose: Arabinose: Xylose:

Galactose: Other Sugars = 61.0: 6.2: 2.5: 0.5: 23.1: 6.7; P-RHAM1), β -mannan (from Carob seed, sodium borohydride-reduced, Man: Gal = 97: 3), medium viscosity galactomannan (from guar, Gal: Man = 38: 62, M_r 380,000 P-GGMMV) and glucomannan low viscosity ('KGM', from konjac, Man: Glu ratio 60: 40, M_r 950,000; P-GLCML). *Tamarindus indica* seed xyloglucan ($M_r \sim 2 \times 10^6$) was donated by Dainippon Pharmaceutical Co. (Osaka, Japan). Cellulose I β donor substrates were obtained from various sources (Table S1). [3 H]XXXGol (standard xyloglucan-oligosaccharide nomenclature [26]), XXXGol-sulphorhodamine (XXXGol-SR; Fig. 1a), prepared as previously described [27],[28], and XXXGol-SR-impregnated papers were from EDIPOS (<http://fry.bio.ed.ac.uk/edipos.html>).

2.2. Preparation of bacterial cellulose I α

Culturing *Komagataeibacter xylinus* (traditionally known as *Acetobacter xylinum* or *Gluconacetobacter xylinus*) for bacterial cellulose production was as previously described [29]. Briefly, the bacteria were grown on solid Hestrin-Schramm (HS) agar medium for 7 d at 30°C and a colony was transferred into 6 ml of fresh HS liquid medium and incubated for 7 d more. Next, 0.5 ml was inoculated into 4.5 ml of fresh liquid medium and incubated 3 d, and 1.5-cm-diameter well-plates were filled with 2 ml of 1:14 inoculum:HS fresh medium and incubated for another 3 d. A pellicle of bacterial cellulose was harvested from the surface of each well, soaked for 10 min in 50% ethanol, boiled in water (2 \times 20 min) and immersed in 0.1 M NaOH at 90°C (2 \times 20 min), which removes organic residues but does not appreciably convert cellulose I to II. Finally, the films were neutralised by water-washing.

2.3. Production of glycol chitin from glycol chitosan

Glycol chitosan was dissolved in water (0.5%, w/v) at 100°C for 20 min. After cooling to 20°C, 100 μ l of collidine/acetic anhydride (2:1) was added to 19.9 ml glycol chitosan solution and incubated on a wheel for 1 d. Then, 4 ml of 1 M NaOH was added, resulting in a pH of \sim 12.6.

After 6 h ~~rotation~~on a rotating wheel, the pH was adjusted to 5.5 with acetic acid. A 20- μ l portion of the products and 20 μ l of untreated glycol chitosan solution (0.16% and 0.46%, w/v) were loaded as spots onto Whatman No. 3 paper; the papers were washed in 70% (v/v) ethanol, dipped through 0.2% (w/v) ninhydrin in acetic acid/acetone (0.5:99.5) and heated at 105°C for 30 min, which stains amino groups.

2.4. Preparation of cellulose hydrogels

Cellulose hydrogels were prepared as previously described [30]. Microcrystalline cellulose was mixed with water and swelled for 2 h at 5°C. Aqueous NaOH was precooled to -6°C, then the swelled cellulose was added (final concentrations 5% w/v in 2 M NaOH) and the mixture was stirred at 1000 rpm at -6°C for 2 h. The solution was poured into cylindrical moulds (3 cm diameter, 0.5 cm height) and kept for 2 h at 50°C; in these conditions cellulose gels irreversibly [31]. Disc-shaped gels were placed in water, which was regularly renewed over several day until a neutral pH was obtained.

2.5. Heterologous protein production and *Equisetum* enzyme extraction

Production of *Ej*HTG in *Pichia pastoris* strain SMD1168H was as described before [18]. Extraction of native enzyme from *Equisetum* followed a previous protocol [24]. In brief, ~100 g freshly harvested tissue was ground in 500 ml ice-cold extraction buffer [0.3 M succinate (Na⁺, pH 5.5) containing 3% (w/v) polyvinylpolypyrrolidone] and after centrifugation (10,000 g, 45 min) the supernatant was stored at -80°C.

2.6. Assaying radioactivity

³H in aqueous solutions and ³H bound to various insoluble cellulosic substrates was quantified by scintillation counting in ScintiSafe 3 scintillation cocktail (Fisher Scientific, Loughborough, UK) or in GoldStar 'O' scintillation cocktail (Meridian, Chesterfield, UK) respectively.

2.7. Radiochemical assay of various transglycanase activities with non-cellulosic donor substrates

Two partially overlapping experiments (a and b) were conducted. In both cases the reaction mixture (final volume 20 µl) contained ~~5-µl~~ 25% (v/v) of filtrate from *Pichia* cultures expressing *EfHTG*, 0.1 M succinate (Na⁺, pH 5.5), 0.1% (w/v) bovine serum albumin (BSA), 1.0 or 2.0 kBq acceptor substrate ([³H]XXXGol) and 0.5% (w/v) donor substrate (xyloglucan or MLG for XET or MXE activity respectively; or alternative polysaccharides for surveying possible novel hetero-transglycanase activities). Mixtures were incubated for ~~24-h~~ or 48 h at 20°C, during which period the reactions are approximately linear, and the enzyme was then denatured by addition of 6 µl of 90% formic acid. XET and MXE products were then dried on Whatman No. 3 paper, to which they hydrogen-bond, washed in running tap-water overnight, and quantified for radiolabelled high-*M_r* products by scintillation-counting. In experiment (a), the products (if any) formed from polysaccharides other than xyloglucan and MLG were washed in 72% ethanol until the supernatant was non-radioactive (removal of free [³H]XXXGol). ³H left in the pellets was quantified by scintillation-counting. In experiment (b), any ³H-labelled polymeric products formed in heterotransglycanase reactions were quantified by a glass fibre blotting method followed by scintillation-counting [32] (Fig 3 a and b respectively).

2.8. Radiochemical assay of heterotransglucanase activity with cellulose as donor (CXE activity)

For CXE assays, various cellulose samples were tested after water-washing and drying ('untreated', cellulose I) or after alkali-pretreatment ('mercerisation', cellulose II). For the latter, cellulosic materials including bacterial cellulose were routinely incubated in 6 M NaOH at 20°C for 4 h, washed 4× in copious tap water, then washed in 5% (v/v) acetic acid for 0.5 h, followed by washing in running tap water for 16 h and dried. This converts cellulose I to cellulose II

[33],[34]. *Equisetum* cellulose was prepared from an alcohol-insoluble residue [35] by removal of hemicelluloses with 6 M NaOH (37°C, 3×1 day), and was thus only available as cellulose II. Each type of cellulose (20–29 mg) was soaked with 20 µl reaction mixture (as described in the previous paragraph) with the addition of 0.1 or 1.0% BSA but lacking any deliberately added donor substrate other than the cellulose. For cellulosic paper from print products (e.g. ~~books~~newspapers), 20-mg pieces lacking ink or colour were used. Controls contained heat-inactivated enzymes and these values were subtracted from the non-mock groups, thus correcting values for any possible unspecific [³H]XXXGol binding.

After the CXE reaction had been stopped (in the linear range) with 6 µl of 90% formic acid, cellulose pieces were washed in 6 M NaOH for 12 h at 20°C, 6 M NaOH for 0.5 h at 100°C, and then in running tap-water overnight, dried and assayed for bound ³H. Besides washing out all unreacted [³H]XXXGol, this alkali treatment would solubilise any XET- or MXE-products potentially formed from endogenous hemicelluloses present in crude *Equisetum* enzyme preparations. Some of the cellulosic substrates were water-washed (freed of unreacted [³H]XXXGol) in dialysis tubes, preventing loss of material due to fibre disintegration.

2.9. Fluorescent dot-blot assay of heterotransglucanase activity with cellulose as donor (CXE activity)

Two methods of attaching XXXGol–SR (acceptor substrate) to cellulose were compared. Native *Equisetum fluviatile* enzyme (precipitated in 30% saturated ammonium sulphate and redissolved at various concentrations in 0.2 M succinate buffer containing 1% BSA) was either (a) pre-mixed with XXXGol–SR and then pipetted onto dry filter paper or (b) pipetted directly on-to dry papers that had previously been impregnated with XXXGol–SR at ~1 µmol/m² such that 4 µl of aqueous solution (giving a ~9-mm diameter spot) would lead to an XXXGol–SR concentration of ~20 µM. In both cases, the paper had been marked out in 96-well format and either pretreated in 6 M NaOH, or not, before application of the enzyme and/or acceptor substrate. In both cases,

The volume of solution applied to each ‘well’ position on the paper was 4 µl. After 12 h enzymic reaction under humid conditions (with the paper tightly pressed between two cellulose acetate stationery sheets), the remaining unreacted XXXGol–SR was washed out with formic acid/ethanol/water (1:1:1) for 1 h, and the paper was re-dried. Covalently bound fluorescent products were photographed under 254-nm UV and then the fluorescence intensity of the spots ~~were~~ quantified in ImageJ. In a test of the stability of the cargo’s attachment to the paper, each paper was then bathed in 6 M NaOH for 12 h, neutralised by briefly rinsing in water, dried, and re-photographed under UV, and the fluorescence intensity of the spot was again quantified as ~~before~~above.

2.10. Determining the minimal NaOH pretreatment required to produce cellulose optimised as a CXE substrate

Whatman No. 1 paper (with a pre-printed grid in 96-well-plate format) was pretreated with 0 – 6 M NaOH at 20°C for various times, then washed with water, slightly acidified with 1% acetic acid, and re-washed in running tap-water for 6 h followed by deionised water, and air-dried. The alkali-pretreated papers were tested as substrates for native HTG (CXE activity) obtained from *Equisetum fluviatile* plants.

2.11. Testing the alkali-stability of HTG-generated MXE, XET and CXE products

Transglucanase reactions were performed with *Pichia*-produced *Ef*HTG as above, with 6 M NaOH-pretreated Whatman No. 1 filter paper (for CXE), MLG (for MXE activity) or xyloglucan (for XET) as donor substrate, and [³H]XXXGol as acceptor. We then tested the alkali-stability of the three radiolabelled products obtained (cellulose–[³H]XXXGol, MLG–[³H]XXXGol and xyloglucan–[³H]XXXGol; washed rid of unreacted [³H]XXXGol with water or 75% ethanol for cellulose and the hemicelluloses respectively). For the CXE product, 0.25-mg pieces (each ~1 kBq) of the cellulose–[³H]XXXGol were incubated at 100°C for 1 h in 100

257 µl of 0–6 M NaOH, then cooled and diluted to 1 ml with water. The solution was centrifuged
 258 (12,000 g for 2 min) and the supernatant was slightly acidified with acetic acid, then assayed for
 259 ^3H ; the pelleted paper was suspended in 2 ml of 5% acetic acid, and likewise assayed for ^3H .
 260 The proportion of the total ^3H (from cellulose- $[\text{}^3\text{H}]\text{XXXGol}$) that remained water-insoluble was
 261 thereby calculated. The water-soluble XET and MXE products (1 kBq in ~4-µl) were mixed
 262 with 100 µl of 0–6 M NaOH, and incubated at 100°C for 1 h. After cooling, 100 µl of 50%
 263 acetic acid was added and 100 µl of the acidified solution was dried onto a 6×4-cm piece of
 264 Whatman No. 3 paper, which was then washed in running tap-water for 72 h, dried and assayed
 265 for hydrogen-bonded $[\text{}^3\text{H}]$ hemicellulose.

The free acceptor substrate was also incubated in various concentrations of NaOH at
 100°C for 1 h, then slightly acidified with acetic acid, dried, redissolved in water and assayed
 for remaining non-volatile (heat-stable) ^3H . Additional samples of $[\text{}^3\text{H}]\text{XXXGol}$ that had been
 treated in 0 and 6 M NaOH were analysed by paper chromatography on Whatman No. 1 in ethyl
 acetate/acetic acid/water (10:5:6) for 40 h. The chromatogram was cut into strips, which were
 assayed for ^3H .

2.12. CXE assays on cellulose hydrogels

Blocks of hydrogel (20 mm³) were equilibrated in 200 mM succinate (Na⁺; pH 5.5), then soaked
 with 30 µl reaction mixture [10 µl native *Equisetum* protein extract or *Pichia*-produced *EfHTG*,
 with additives to give a final composition of 133 mM succinate buffer (Na⁺; pH 5.5) containing
 1 kBq $[\text{}^3\text{H}]\text{XXXGol}$, 1.0% BSA and 0.5% chlorobutanol] and incubated for two weeks at 20°C
 (linear reaction range). After the reaction had been stopped, the hydrogel was washed 3 × in
 water, then heated in 2 M TFA at 120°C for 1 h. The ^3H in the TFA supernatant and pelleted
 cellulose were separately quantified by scintillation counting.

To visualise whether *Pichia*-produced *EfHTG* can form CXE products inside cellulose
 hydrogel blocks, we used a 20-mm³ piece of buffer-equilibrated gel (2×2×5 mm) to which was

added 10 µl of enzyme solution plus 10 µl of 7.2 µM XXXGol–SR. Thus, if uniformly distributed within the gel, the XXXGol–SR concentration was 1.6 µM. Controls lacked the enzyme. After the reaction had been stopped at 72 h, the gel blocks were rinsed for 3 days in water, then sectioned with a razor blade (giving a slice ~2×5×0.6 mm) and examined with a Leica DM2000 LED microscope equipped with a Leica DFC7000 T camera and Leica EL6000 external light source. Incorporated SR was visualised with a GFP filter cube (excitation band pass (BP) 470/40 nm, emission BP 525/50 nm) using LAS X software. Minimal adjustments to contrast were applied equally across entire image plates.

3. Results

3.1. *Ef*HTG acts on a wide range of cellulosic donor substrates derived from plants and bacteria

So far, *Ef*HTG's CXE activity had only been tested on cotton cellulose I β (Whatman No. 1 filter paper) [18],[36]. To explore whether *Ef*HTG can act on other forms of cellulose, we tested a variety of papers and other commercial cellulosic products, which had undergone different industrial treatments (Table S1). Each cellulose sample was used with and without a pretreatment in 6 M NaOH. Exposure to NaOH turns the cellulose I crystal structure to Na-cellulose; water washing converts it to cellulose II hydrate and the following drying yields cellulose II [37]. NaOH treatment and conversion to cellulose II may cause a partial cellulose depolymerisation by oxidation and subsequent β -alkoxy-elimination [38],[39].

*Ef*HTG acted on most cellulose samples tested, but at different rates (Fig. 1b). Of plant-derived cellulose samples that were not pretreated with alkali, the most effective donor substrates were less-processed papers (paper napkins, handkerchieves, toilet paper, *The Sun* newspaper, a glossy brochure and Whatman filter papers), whereas the least effective included cotton wool, cotton fabric (lab-coat), absorbent sanitary products, and certain papers (*The Guardian* newspaper, lens-cleaning tissue, printer paper, thin brochure paper and lab-book paper).

The highly modified cellulose derivative, nitrocellulose, was not an effective donor substrate for HTG, as previously reported for other cellulose derivatives such as carboxymethylcellulose [24] and water-soluble cellulose acetate [18].

All substrates based on plant-derived, unsubstituted cellulose supported higher CXE activities after pretreatment with 6 M NaOH (and thus presumably converted to the cellulose II allomorph; Fig. 1b). There was no simple relationship between the substrate effectiveness after alkali-pretreatment and that observed without NaOH pretreatment. Therefore, there was wide variation in fold-stimulation by alkali pretreatment: for example, the glossy brochure paper was

enhanced only 1.8-fold, whereas Whatman No. 1 filter paper was enhanced 13.5-fold, and some samples (e.g. printer paper and cotton wool) were enhanced over 60-fold.

Only bacterial cellulose I α , which differs conformationally from the cellulose I β predominating in plants [40], served as a better substrate in its native form than after pretreatment with 6 M NaOH for 4 h. Although 6 M NaOH converts bacterial cellulose I α to cellulose II within 3 min, complete mercerisation takes longer than for I β as the interdigitation between microfibrils is different and the crystallinity is higher than in plant-derived cellulose [34],[41]; this may have been responsible for our observed decrease in substrate effectiveness. Indeed, native bacterial cellulose I α , despite its high crystallinity, yielded even more CXE product than the non-alkali-pretreated cellulose I β of Whatman filter papers (Fig. 1b). The native bacterial cellulose I α as used had been freed of contaminants by a brief treatment with 0.1 M NaOH at about 90°C; these alkaline conditions are too mild to produce appreciable amounts of cellulose II [40]. Pretreating the bacterial cellulose ~~it~~ with 6 M NaOH at 20°C caused the never-dried bacterial cellulose to shrink drastically (Fig. 1b), as also reported previously [34].

3.2. Alkali pretreatment enhances the donor substrate effectiveness instantaneously but requires ≥ 3 M NaOH

We next investigated the NaOH concentration and duration required for enhancing plant-derived cellulose as a donor substrate. Alkali pretreatment of (cotton-derived) Whatman No. 1 filter paper at 20°C increased the subsequently observed CXE reaction rate. The minimal effective alkali concentration for this was 3 M NaOH (Fig. 2; Fig. S1), which agrees with the cut-off reported for the conversion of plant cellulose I to cellulose II by NaOH [42]. The same is true for the conversion of bacterial cellulose I to cellulose II by NaOH at 22°C [34]. With 3–6 M NaOH, the improvement of the donor substrate for CXE activity (presumed to be due to formation of the type II cellulose allomorph) at 20°C occurred in < 4 min — thus essentially instantaneously — and did not appreciably increase during the next 16 h (Fig. S1). The I \rightarrow II

conversion of bacterial cellulose by 6.25 M NaOH at 22°C was ~~even~~ complete within 3 min as was also reported before [34]. The measured CXE activity was highest on papers pretreated with 4.5 or 6.0 M NaOH.

In the experiments shown in Fig. 2 and Fig. S1, conducted with native *Equisetum* enzyme, the optimal NaOH pretreatment led to a 1.8–2.5-fold increase in CXE activity. This is substantially less than the 13.5-fold increase seen with *Pichia*-produced *Ef*HTG (Fig. 1b, dark bar referring to Whatman No 1). We suggest that this difference between *Pichia*-produced *Ef*HTG and the native *Equisetum* extract is because the latter contains not only HTG but also expansins, hydrogen-bond-cleaving proteins which, to some extent, mimic NaOH in disrupting the conformation of cellulose I. Added expansins have indeed been found to enhance the CXE action of *Pichia*-produced *Ef*HTG on alkali-nonpretreated cellulose [36], presumably due to weakening the lateral adhesion of load-bearing polysaccharides to the cellulose surface.

3.3. Dot-blot method for covalently attaching a fluorescent ‘cargo’ to filter paper

Two methods of attaching a fluorescently labelled xyloglucan-oligosaccharide–sulphorhodamine conjugate (XXXGol–SR), as acceptor substrate, to cellulose were compared. Native *Equisetum fluviatile* enzyme (at various concentrations) was either (a) pre-mixed with XXXGol–SR and then pipetted onto filter paper or (b) pipetted directly onto dry papers which had previously been impregnated with XXXGol–SR. (In both cases, the paper had been either alkali-pretreated, or not, before application of enzyme and/or acceptor substrate.) After 12 h enzymic reaction under humid conditions, covalently attached XXXGol–SR was monitored by its fluorescence.

All enzyme concentrations tested achieved high incorporation of fluorescence under both regimes (a and b), showing that the enzyme was highly active (Fig. S2a). The highest enzyme concentrations produced a fluorescent ring rather than a uniformly fluorescent disc, probably because the high protein concentration, which remained close to the point of application on the

paper, partially quenched the sulphorhodamine's fluorescence. This was particularly the case in method (b), where the applied enzyme solution would have carried some of the previously applied-impregnated XXXGol–SR towards the periphery of the blot, leaving a ring-shaped 'tide-mark'. Image analysis showed that this ring-shaped fluorescence signal at high enzyme concentrations resulted, for most samples, in a slightly lower total fluorescence intensity of spots than with the signal at lower enzyme loadings, probably owing to quenching of sulphorhodamine's fluorescence by proteins-(Fig. S2b). Advantageously, this suggests that a high and evenly distributed cargo incorporation can be achieved with relatively small amounts of enzyme. As expected, the alkali-pretreated papers (cellulose II) gave more intense spots of product (Fig. S2). Thus, both regimes (a and b) are valuable — (a) giving more uniformly fluorescent discs, and (b) being preferred for conveniently testing diverse enzyme preparations.

Moreover, the fluorescent cellulose–XXXGol–cargo linkage was largely stable upon further harsh alkali treatment (Fig. S2): 60–80% of fluorescence was retained after post-treatment in 6 M NaOH for 12 h. Papers not pretreated with alkali retained slightly more fluorescence: ~75–80% (not pretreated) vs. ~60–70% (pretreated).

3.4. Cellulose hydrogel as a donor substrate

We also tested a cellulose hydrogel, which is a swelled, hydrophilic network built of coagulated cellulose II and pores filled with water. Hydrogels' dry counterparts, aerogels, are of low density (around 0.1 g/cm³ [30]); the fraction of the pores' volume in a hydrogel is thus above about 90%. The size of the pores in cellulose aerogels varies from a few tens of nanometres to a few micrometres [30], and we presume they are similar in cellulose hydrogels. *Ef*HTG proved able to incorporate the fluorescently labelled acceptor substrate XXXGol–SR deeply into the gel structure (Fig. 1c), showing that the enzyme was able to permeate the hydrogel. Incorporation into the hydrogel was confirmed quantitatively with [³H]XXXGol as acceptor substrate (Fig. 1d). A fraction of the ³H-labelled CXE hydrogel products even resisted hot TFA treatment (Fig.

1d), which might have been expected to hydrolyse the XXXGol moiety of cellulose–
[³H]XXXGol if it behaved the same as free [³H]XXXGol. This observation confirms that the
enzyme can deeply penetrate the hydrogel.

3.5. Range of non-cellulosic donor substrates

After testing numerous cellulosic donor substrates, we explored whether a set of different
polysaccharides can serve as donor substrates. We confirmed that two plant-derived
hemicelluloses, xyloglucan and MLG, are good donor substrates (XET and MXE activity,
respectively; Fig. 3a,b). Lichenan, which is a lichen-sourced MLG with very few cellotetraose
sequences, was slightly active, as reported before [18]. However, the other plant-derived
hemicelluloses — including β -mannan, glucomannan, galactomannan, arabinogalactan,
glucuronoxylan and laminarin (an algal polysaccharide which chemically resembles plant
callose) — did not support any appreciable activity compared with xyloglucan or MLG (Fig.
3a,b). One commercial xylan preparation from oat exhibited some activity (~5% of XET
activity; Fig. 3a), which, however, probably resulted from contamination by commercial oat
MLG since birch xylan was not a substrate. Other xylan preparations did not serve as donor
substrate (Fig. 3a,b). Various other polysaccharides were almost inactive, including plant pectic
components, seaweed polymers, and yeast α -mannan (Fig. 3b).

Interestingly, however, various commercial preparations of chitosan [(1→4)-linked
polysaccharide of β -D-glucosamine] consistently generated ethanol-insoluble transglycanase
products (chitosan–[³H]XXXGol conjugates), amounting to ~10% of the observed XET activity
(Fig. 3a). The degree of acetylation in chitosan (a polymer of non-acetylated β -(1→4)-D-
glucosamine) preparations (~75–85%) did not affect their suitability as a donor substrate (Fig.
3a). In contrast, chitin [(1→4)-linked polysaccharide of *N*-acetyl- β -D-glucosamine] did not
serve as a transglycanase substrate (Fig. 3a). However, water-soluble glycol chitin, which we

produced by *N*-acetylating commercially available glycol chitosan (Fig. 3c). ~~Efficient *N*-acetylation was confirmed by the absence of ninhydrin staining in glycol chitin preparations (Fig. 3e). Glycol chitin~~ was utilised as a donor substrate (activity was up to ~8% of XET activity; Fig. 3d). Glycol chitosan even supported a transglycosylation rate of up to ~15% of XET activity (Fig. 3d). Efficient *N*-acetylation was confirmed by the absence of ninhydrin staining in glycol chitin preparations (Fig. 3c).

3.6. Cellulose–xyloglucan bonds are highly stable in hot alkali

For the polysaccharide–xyloglucan bonds of the hetero-transglucanase products to be valuable industrially, they need to be chemically robust. We tested this by incubating cellulose–³H]XXXGol, MLG–³H]XXXGol and xyloglucan–³H]XXXGol conjugates (i.e., CXE, MXE and XET products respectively) in 0.1–6.0 M NaOH at 100°C for 1 h. Even after heating in the most severe alkali tested (6 M NaOH; pH > 14.7), over 80% of the radioactivity remained associated with the respective polysaccharide in each case (Fig. 4a) — as judged by water-insolubility (cellulose–³H]XXXGol) or by retention of the ability to hydrogen-bond to paper (both the hemicellulose–³H]XXXGol conjugates). The free ³H]XXXGol oligosaccharide itself showed a similar stability, with > 80% of the radioactivity remaining non-volatile, and thus not exchanged as volatile ³H₂O (Fig. 4a). We therefore suggest that the apparent loss of polysaccharide–XXXGol was mainly due to the slight instability of the C-1–³H bond within XXXGol rather than to any instability of the polysaccharide–XXXGol glycosidic bond.

This idea was supported by the observation that ³H]XXXGol retained its original mobility on paper chromatography (Fig. 4b). Although ~14% of the ³H became volatile (presumably as ³H₂O) after heating under the most extreme alkaline conditions, the remaining non-volatile material maintained the standard *R_F* of a heptasaccharide (³H]XXXGol), indicating that glycosidic bonds were not appreciably cleaved.

4. Discussion

4.1. Enzymic covalent modification of cellulose

Hetero-trans- β -glucanase (*Ef*HTG) can catalyse the formation of covalent bonds between insoluble cellulose and soluble xyloglucan oligosaccharides [18]. Here we show that the enzyme is effective on broad range of cellulose substrates preparations(Fig. 1b, Fig. 3a+b), and that in all plant-derived celluloses studied the enzymic reaction is enhanced if the cellulose is pre-converted from the I β to the II allomorph (shown diagrammatically in Fig. 5a). Another factor improving NaOH-treated cellulose as a CXE substrate might be alkaline ‘peeling’ (progressive removal of glucose moieties from the reducing end in the form of saccharinic acids), but mid-chain cellulose hydrolysis does not occur at room temperature [43]. Potentially, peeling could loosen up the cellulose network and make more cellulose chains accessible for enzymic modification, explaining higher CXE activities after NaOH exposure. However, treatment with 4.5 M NaOH (~5 min) at room temperature was optimal (Fig. 2) and it is unlikely that appreciable cellulose degradation occurs under such conditions [44]. Increasing the NaOH concentration to 6 M and the treatment time to 4 h did not further enhance the cellulose as a substrate (Fig. 2). We thus conclude that the better suitability of plant derived-celluloses after NaOH treatment is primarily due to cellulose I to cellulose II conversion.

The covalent cellulose–XGO bonds formed are highly stable, resisting solubilisation even in boiling alkali (Fig. 4a) and partially withstanding hot acid (Fig. 1d). The process of loading cellulose with a chemical ‘cargo’, potentially transferrable to the biotechnology industry, is illustrated in Fig. 5b.

A cCellulose-acting transglucanase activity for the *Arabidopsis* AtXTH3 protein has been shown before.¹⁹ However, AtXTH3 showed appreciable CXE activity only with cellulose that had been rendered amorphous by pretreatment with phosphoric acid, and not with microcrystalline cellulose. Moreover, CXE is not the major activity of AtXTH3, representing only ~45% of its predominant XET activity.

Here we confirm that *Ej*HTG is preferentially a hetero-transglucanase (its CXE activity is 200–300% of its XET activity [18]), and we show that it can act on celluloses with various degrees of crystallinity. This includes the cellulose of cotton ‘fibres’ (specialised seed trichomes; crystallinity ~60% [45]) and bacterial cellulose I α . Interestingly, the native bacterial cellulose of biofilms functioned as a better CXE donor substrate than all untreated plant-derived cellulose samples tested, including Whatman filter paper, which is manufactured from cotton (Fig. 1b). Cellulose biofilms produced by *Komagataeibacter xylinus* are dominated by cellulose I α and exhibit a higher crystallinity (~75–95%, depending on the method used [41],[46]), M_r and water-holding capacity than land-plant cellulose [45]. The crystalline fraction of the latter is dominated by cellulose I β . In both crystal forms, cellulose chains form flat ribbons, where glucose monomers are locked in position by hydrogen-bonds between –OH groups and ring-O atoms [47]. In a cellulose microfibril, these chains lie parallel and form hydrogen-bonds edge-to-edge, creating sheets, which are stacked onto each other and held in place via weak C–H to O hydrogen-bonding [48].

The main reason for the different properties of cellulose I α and I β is the relative displacement of the sheets in the chain direction [48]. The very high water-holding capacity of bacterial cellulose (more than 100 times its dry weight [40]; compared with 2.0 ± 0.1 times for Whatman paper No. 1 paper), suggests an easy accessibility of cellulose chains and might explain its high suitability as a CXE donor substrate. The water holding capacity of bacterial cellulose remains considerably higher than that of paper even after drying [49]. In contrast to the apparently open structure of bacterial cellulose, NaOH pretreatment caused its dramatic compaction (Fig. 1b), diminishing its ability to absorb water and consequently its suitability as a substrate. For all other celluloses tested, NaOH-pretreatment, which converts cellulose I into the allomorph cellulose II with antiparallel arrangement of cellulose chains, consistently increased the CXE activity catalysed by *Ej*HTG. Still, several tested cellulosic substrates (e.g. newspaper

or filter papers) supported high CXE activity without NaOH pretreatment, suggesting differences in accessibility.

A priori, it might be postulated that our 6 M NaOH pretreatments washed out various industrial additives (e.g. other polysaccharides, sizing agents and inorganics) from some of the cellulose samples and that this purification is what enhanced the donor substrate effectiveness for CXE activity. However, the cellulosic substrates that were not NaOH-pretreated were always washed in water, which would be expected to remove ~~some~~ the main contaminants. Furthermore, pure paper (e.g. Whatman filter papers) always supported much higher CXE activity when pretreated with NaOH, suggesting that the alkali effect is due to conversion of cellulose I to II, which should apply to all papers. For printed paper (e.g. newsprint) we always used unprinted areas.

4.2. Potential biotechnological applications

As discussed above, *Ef*HTG is a predominant hetero-transglucanase that grafts various kinds of insoluble cellulose (donor substrate) onto xyloglucan oligosaccharides (acceptor substrate). We anticipate several industrial processes where HTG might be applied to produce cellulose-based products with new properties and functionality. The xyloglucan oligosaccharide can easily be loaded with a valuable cargo (dye, tracer molecule, indicator, drug etc.) at the hemiacetal group of its reducing terminus. This group is far removed from the –OH group at C-4 of the oligosaccharide's non-reducing terminal glucose residue, which is the site selected by *Ef*HTG as its acceptor substrate; therefore the 'cargo' does not sterically block the hetero-transglycosylation reaction. We illustrated this by showing that both the smallest possible cargo (tritium) as well as a 'bulky' fluorophore (sulphorhodamine; Fig. 1a) can be efficiently incorporated into e.g. paper (Fig. 1b, Fig. S2). Consequently, *Ef*HTG can be used to attach various cargoes to commercial celluloses without the use of toxic or environmentally harmful chemical treatments.

Since plant cellulose is the world's most abundant organic substance, numerous biotechnological applications can be envisaged for bulk cellulosic materials (wood-pulp, cotton fabrics, linen, etc.), for example in the manufacture of paper, tissue, sanitary materials, packaging and textile industries. Among numerous other potential examples, the covalently introduced 'cargoes' could be fluorophores such as the sulphorhodamine used in the present work (Fig. S2), which would be valuable for publicly invisible quality control. Other cargoes could include visible dyes and various indicators. For instance, in food-packaging materials, covalently bound indicators that would not 'leak out' could report the contained foodstuff's pH and its history of exposure to cumulative temperature/time status, oxygen, redox status, salts or toxic metals, biogenic amines and sulphides (formed from proteins in food that is spoilt), antibodies (potentially revealing undesirable microbes or their by-products, e.g. aflatoxins). Essentially any indicator that is available as a 'dipstick' (such as ~~such as~~ pH paper, indicator paper and test strips for water quality assessment) could in principle be permanently (covalently and chemically stably) bonded to cellulosic packaging by *E/*HTG. Other cargoes could include antibiotics, drugs, disinfectants, positively and negatively charged groups, and tamper-proof markings. The covalent attachment of such cargoes to cellulosic products can be achieved by use of *E/*HTG via a low-cost procedure, using reagents likely to be acceptable in food and medical applications. Furthermore, mixing cargoes would allow the attachment of multiple groups to the cellulosic material in a single step, e.g. allowing the production of universal pH indicator strips, where the blended indicator dyes do not leak out. The cellulose–oligosaccharide–cargo product can withstand remarkably harsh treatments, e.g. with hot, concentrated alkali (Fig. 1b, Fig. 4a, Fig. S2) or even hot acid (Fig. 1d). HTG-modified cellulosic materials would be expected to be less environmentally persistent than fossil fuel-based plastics, and potentially replace them in some applications.

Not only bulk sources of cellulose, but also cellulosic hydrogels and bacterial cellulose are suitable resources for small-scale, high-technology applications, for instance in medicine and

pharmacy [50],[51], or for manufacturing chromatographic matrices. Hetero-transglucosylation could provide a cheap and environmentally friendly method to functionalise inert cellulosic substrates, e.g. introducing xyloglucan-oligosaccharide-conjugated antibiotics into wound dressings [52] or personal protection equipment. The newly discovered ability of HTG to act on chitin and chitosan may also offer biomedical applications where these polysaccharides are widely used.

5. Conclusion

This paper deals with cellulose and other plant cell-wall polysaccharides — sustainable, degradable ~~polymers based on natural~~ and renewable resources, — and explores specifically these polymers² their enzymic functionalisation. conjugates and the creation This has high potential to create various of bioactive cellulose or chitin surfaces ~~es~~ and hydrogels. We found that a hetero-transglucanase acts on most plant-derived cellulose I β substrates and on bacterial cellulose I α , as well as on cellulose II including that in the form of a hydrogel. This offers great biotechnological potential, allowing the covalent grafting of cellulose to (oligo)xyloglucans, offering a novel ‘green’ ~~method~~ technology of functionalising cellulose and other biomass components. We pioneer the enzyme-based functionalisation of cellulose for commercial exploitation by exploring HTG’s range of natural substrates, discovering ~~some~~ new commercially intriguing examples (chitin and chitosan), and we characterise the enzyme’s ability to utilise diverse commercial cellulose-based substrates ~~preparations of cellulose~~, including papers, pulps, textiles, hydrogels and bacterial pellicles, and revealing an unexpected difference between cellulose allomorphs I α and I β . We also report on the very high stability of the functionalised cellulose conjugates that HTG generates, especially their high resistance to hot, concentrated alkali. These conjugates can carry industrially valuable ‘cargoes’, thus providing a biotechnological tool to manufacture commercial cellulose-based products useful in fabrics, pulps, papers, packaging, sanitary and medical products, and hydrogels.

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Declaration of competing interest

The authors declare that they have no conflict of interest. A patent application (WO2015044209) has been filed by BASF and The University of Edinburgh for the use of hetero-transglycosylase. L.F., F.M., A.H., and S.C.F. are inventors.

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Abbreviations

BSA, bovine serum albumin; CXE, cellulose:xyloglucan endotransglucosylase; HTG, hetero-
trans- β -glucanase; MLG, mixed-linkage β -D-glucan; MXE, MLG:xyloglucan
endotransglucosylase; SR, sulphorhodamine; TFA, trifluoroacetic acid; XET, xyloglucan
endotransglucosylase (homotransglucanase); XGO, xyloglucan oligosaccharide; XTH,
xyloglucan endotransglucosylase/hydrolase; XXXGol, reduced heptasaccharide of xyloglucan
(xylose₃.glucose₃.glucitol)

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Figure captions

Fig. 1. Activities of *Ef*HTG on insoluble cellulosic donor substrates. (a) Two Glc₄-based xyloglucan oligosaccharides used as acceptor substrates: [³H]XXXGol and XXXGol—SR. R₁, tritium-labelled glucitol moiety; R₂, sulphorhodamine-labelled glucitol moiety; (b) CXE activity on various plant-derived cellulosic substrates and bacterial cellulose pretreated, or not, with 6 M NaOH. “X” indicates that this sample was not measured (*Equisetum* cellulose was available only as cellulose II as it is not possible to isolate hemicellulose-free cellulose without prior alkali treatment); n=3±SD. Insets show bacterial cellulose films in water before (–NaOH) and after (+NaOH) treatment with 6 M NaOH; scale bar = 1 cm. Acceptor substrate, 1 kBq [³H]XXXGol; enzyme, *Pichia*-produced *Ef*HTG. (c) XXXGol–SR (acceptor substrate) incorporation by *Ef*HTG into 20-mm³ pieces of hydrogel (approximately 2×2×5 mm), as visualised in cross-section by fluorescence microscopy; scale-bar = 2 mm. Above, with enzyme; below, without. (d) CXE activity on cellulose hydrogels plus [³H]XXXGol; the formed products were separated into a hot TFA-extractable fraction (SN TFA) and a non-extractable fraction (pellet TFA); n=2±SD. Enzyme: *Pichia*-produced *Ef*HTG or native HTG extracted from *Equisetum fluviatile* plants.

Fig. 2. Estimating the duration and concentration of alkali treatment required to improve the donor efficiency of cellulose. CXE reactions were set up with variously NaOH-pretreated samples of Whatman No. 1 paper (~29 mg) as donor substrate, and 2 kBq [³H]XXXGol as acceptor, in the presence of 1% BSA. The enzyme was a native *Equisetum fluviatile* extract (precipitated in 30% saturated ammonium sulphate) and the enzymic reaction time was 0.5 h. The donor substrate had been pretreated with 0–6 M NaOH, for 4 min to 4 h as shown on the x-axis. The CXE activity is quoted per unit of enzyme extracted from 1 g fresh weight of *Equisetum* tissue per 0.5 h.

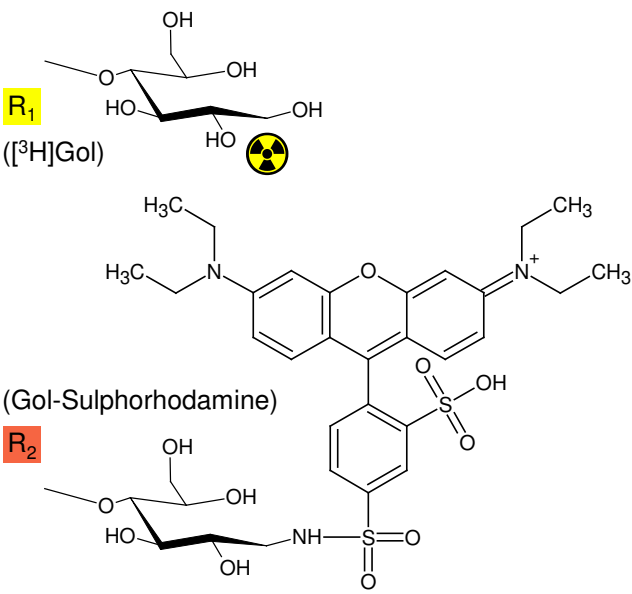
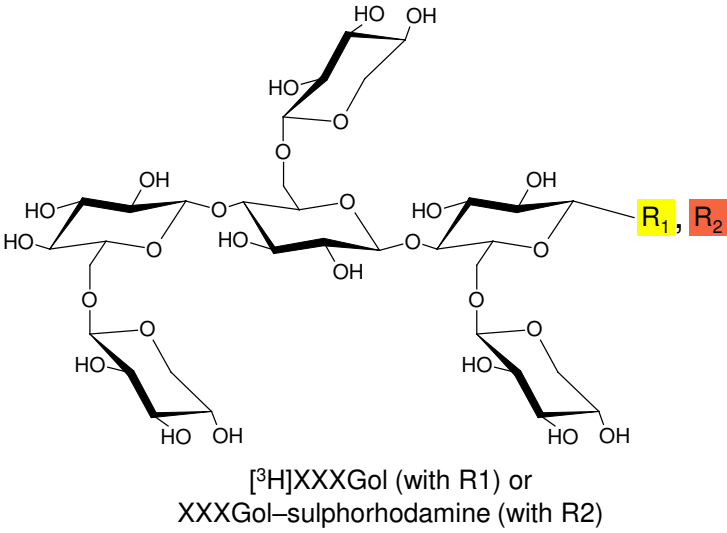
Fig. 3. Testing transglycanase activity on various non-cellulosic polysaccharides. The reaction mixtures contained 0.5% ‘donor’ polysaccharide, 1 or 2 kBq [^3H]XXXGol as acceptor substrate, *Pichia*-produced *Ef*HTG and 0.1% BSA. All values are corrected for a zero-enzyme control. (a) Experiment a: radioactive products harvested by ethanol precipitation; 1 kBq acceptor; $n=4\pm\text{SD}$. (b) Experiment b: products harvested by the glass-fibre blotting method; 2 kBq acceptor; $n=3\pm\text{SD}$ [the ‘control’ shown on the x -axis had enzyme but no donor substrate ($n=8\pm\text{SD}$)]. Abbreviations: l.v., m.v., h.v. = low-, medium- and high-viscosity mixed-linkage β -glucan. (c) Glycol chitosan (characterised by a higher degree of ninhydrin-stainable free amino groups, arrows) and glycol chitin (with acetylated amino groups) were both prepared at 0.16% and 0.46% (w/v), as indicated, and spotted on to filter paper (plusses vs. minuses). (d) Donor substrates: soluble glycol chitin or glycol chitosan; data expressed as a percentage of the same enzyme’s XET activity. Absolute XET values are shown above columns in $\text{kcpm} / 24 \text{ h} \pm \text{SD}$; $n=4$. Enzyme: *Pichia*-produced *Ef*HTG.

Fig. 4. Stability of CXE, MXE and XET products in hot alkali. (a) Radiolabelled transglycanase products were manufactured *in vitro* with alkali-pretreated filter paper, MLG or xyloglucan as donor substrates for CXE, MXE and XET activities respectively, using *Pichia*-produced *Ef*HTG as the enzyme. The polymeric products, freed of unreacted [^3H]XXXGol, were then treated with 0.1–6.0 M NaOH at 100°C for 1 h; controls received hot or cold water. The CXE product was monitored for remaining insoluble radioactivity (thus cellulose); soluble MXE and XET products were monitored for their continued ability to hydrogen-bond to paper (thus polymeric hemicelluloses). Free [^3H]XXXGol was also treated with alkali as above and monitored for remaining non-volatile radioactivity (thus not exchanged as $^3\text{H}_2\text{O}$). Solid lines are linear regressions on the log scale. The structural diagrams use the following symbols: blue circles, β -Glc; yellow circles, β -Gal; orange stars, α -Xyl; pink zigzag, [^3H]glucitol. (b) Paper chromatography of free [^3H]XXXGol after alkali treatment. [^3H]XXXGol was treated with 6 M

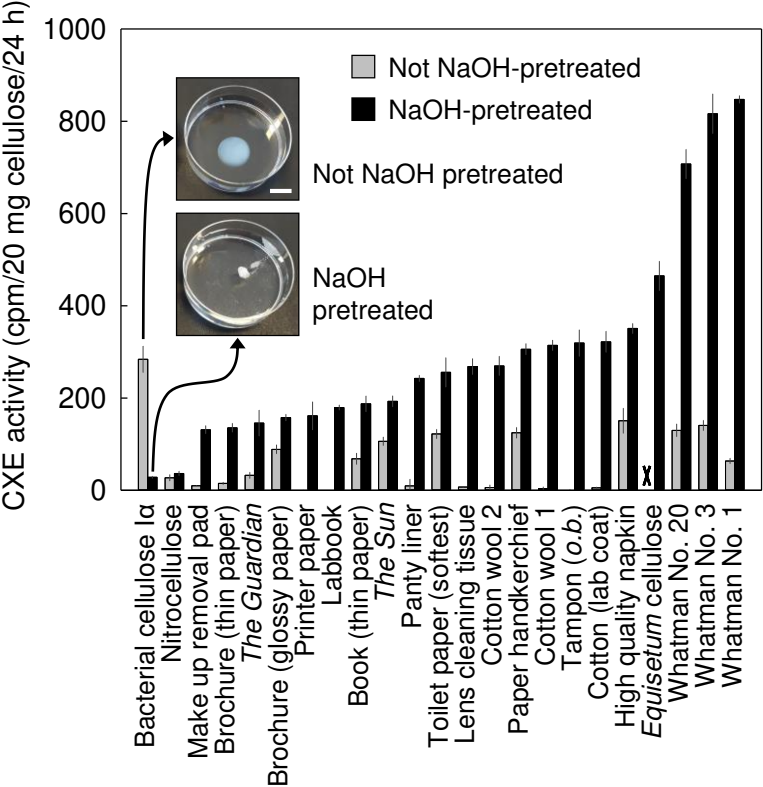
NaOH at 100°C for 1 h, and the remaining non-volatile material was run by chromatography on Whatman No. 1 paper in ethyl acetate/acetic acid/water (10:5:6) for 40 h (black profile). A control received cold water (red line). Glucose, maltose and maltoheptaose (markers) were stained with AgNO₃.

Fig. 5. Interpretation of the observed processes. (a) Cellulose allomorphs before and after mercerisation in alkali. Left: untreated filter paper with cellulose I; 70–80% ordered (crystalline); 20–30% less ordered (amorphous [42]). Right: mercerised filter paper with cellulose II; less ordered, ~60% crystalline; thermodynamically more stable, with inter-sheet hydrogen bonding. (b) Cellulose heterotransglucosylation by *Ef*HTG as a new tool to load a ‘cargo’ stably onto cellulose. The cargo (e.g. a dye) can be covalently attached to the cellulosic substrate (e.g. paper, cellulose hydrogel) at random positions via a xyloglucan oligosaccharide (XXXGol) linker. Glucose residues of cellulose involved in breakage and re-formation of bonds are marked with stars.

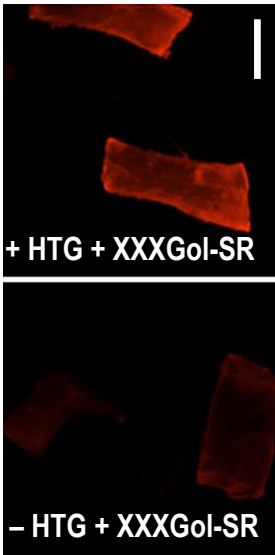
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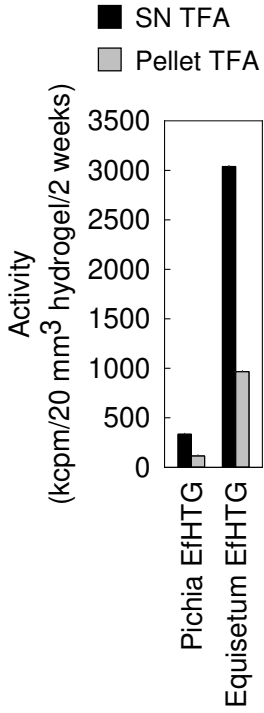
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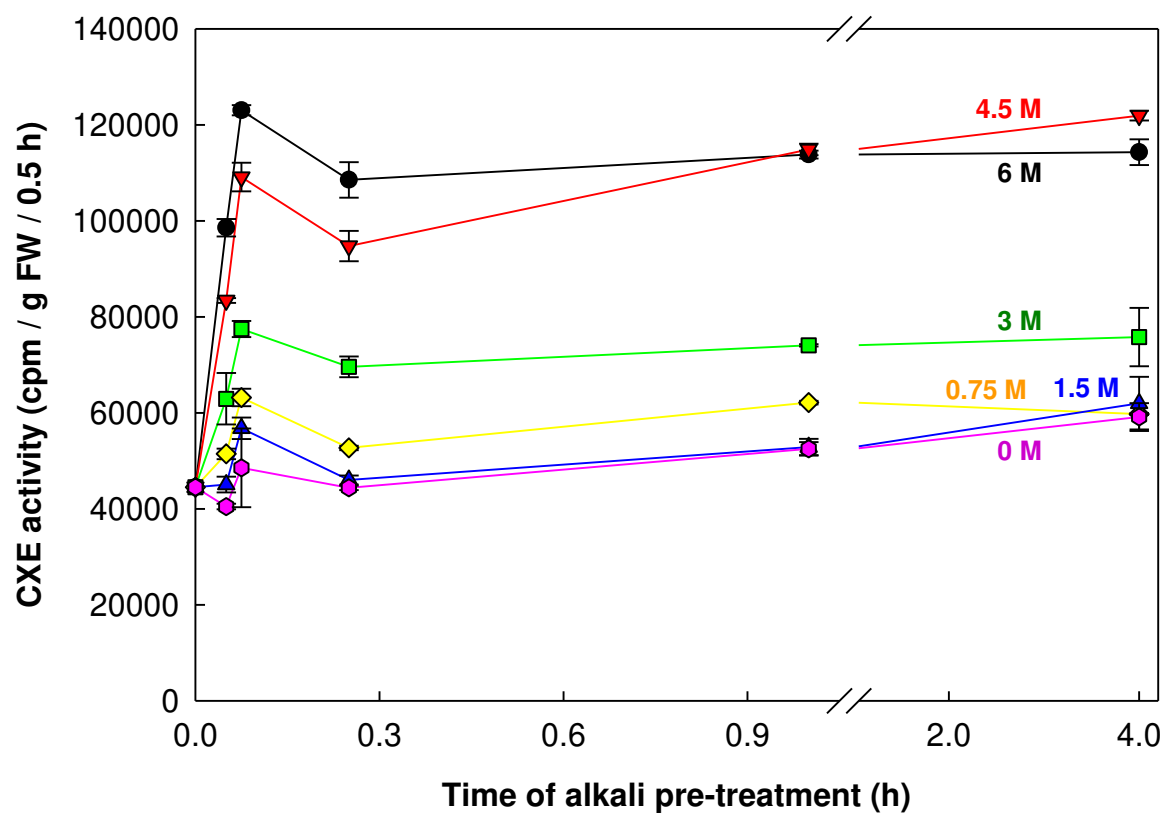
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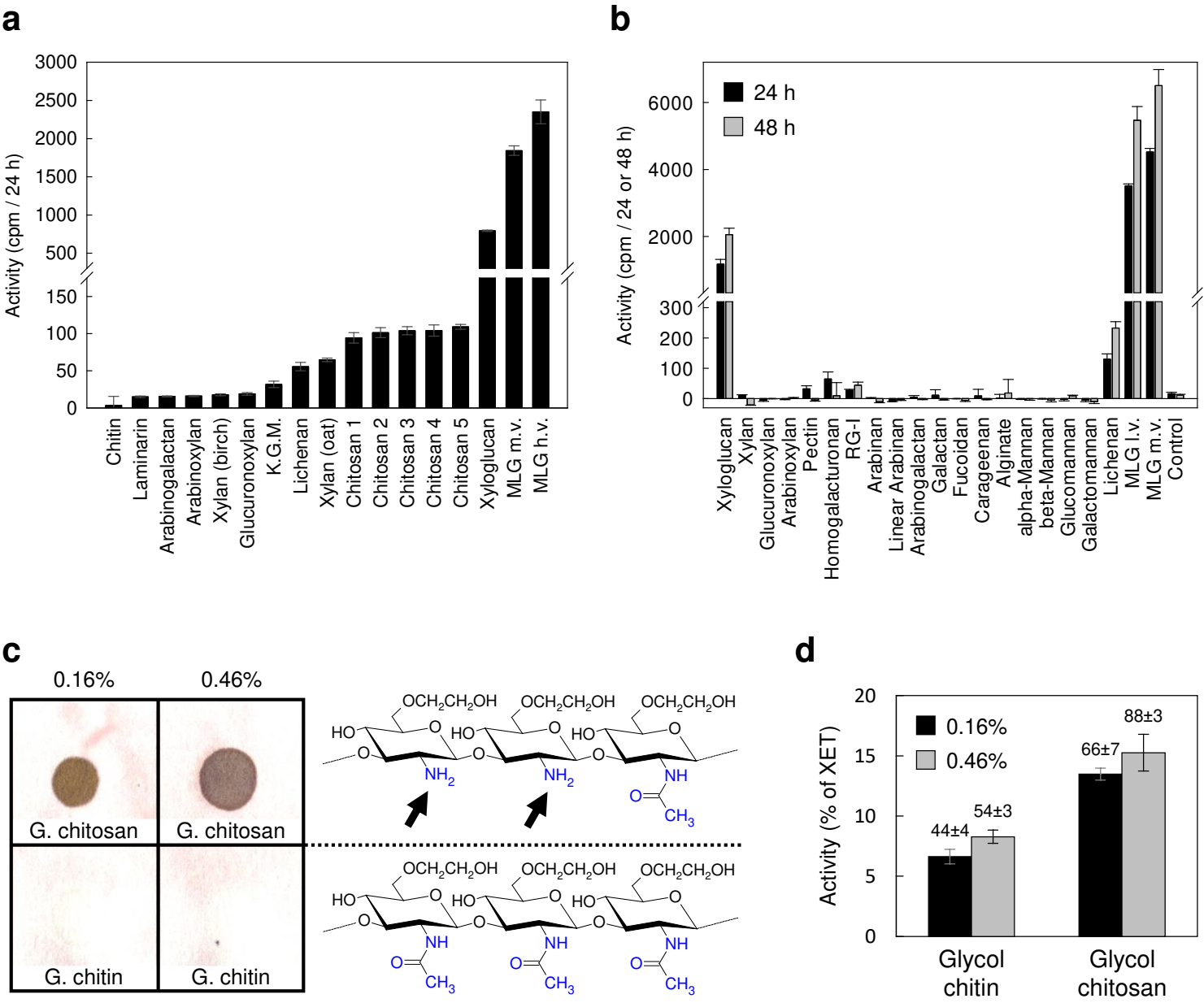


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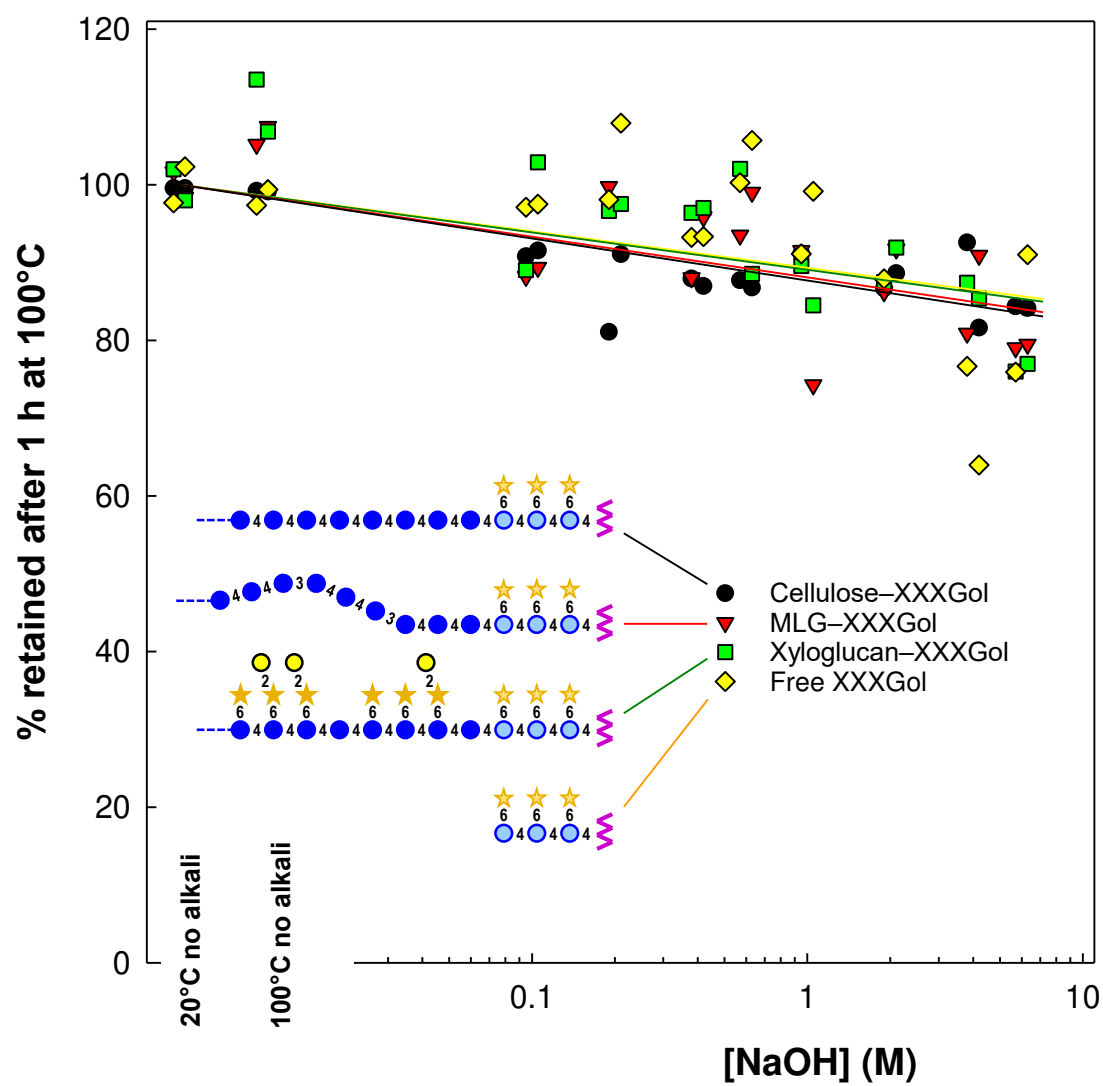
Fig_2



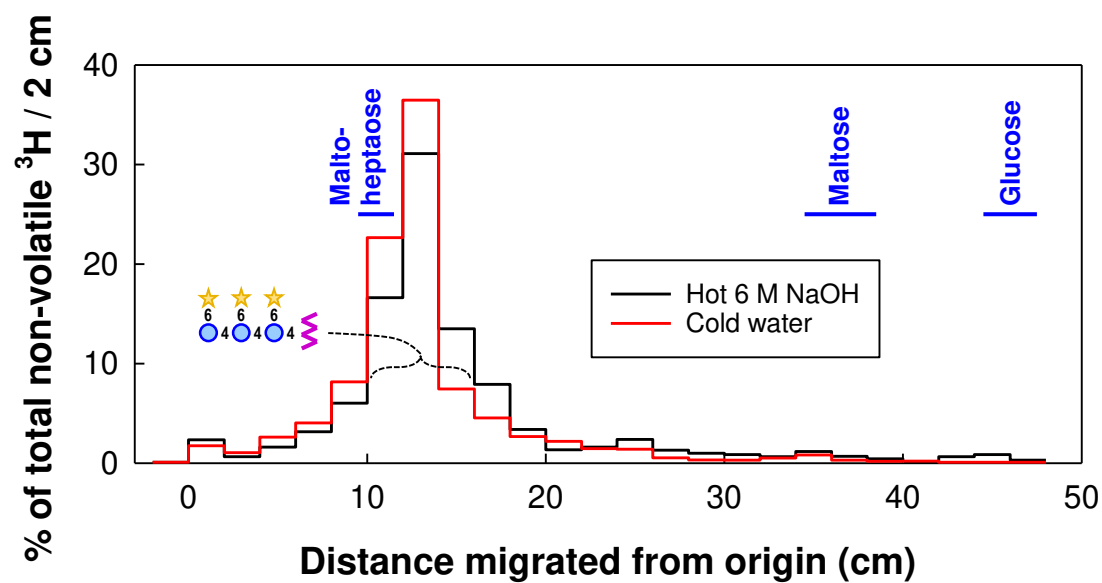


Fig_4

a

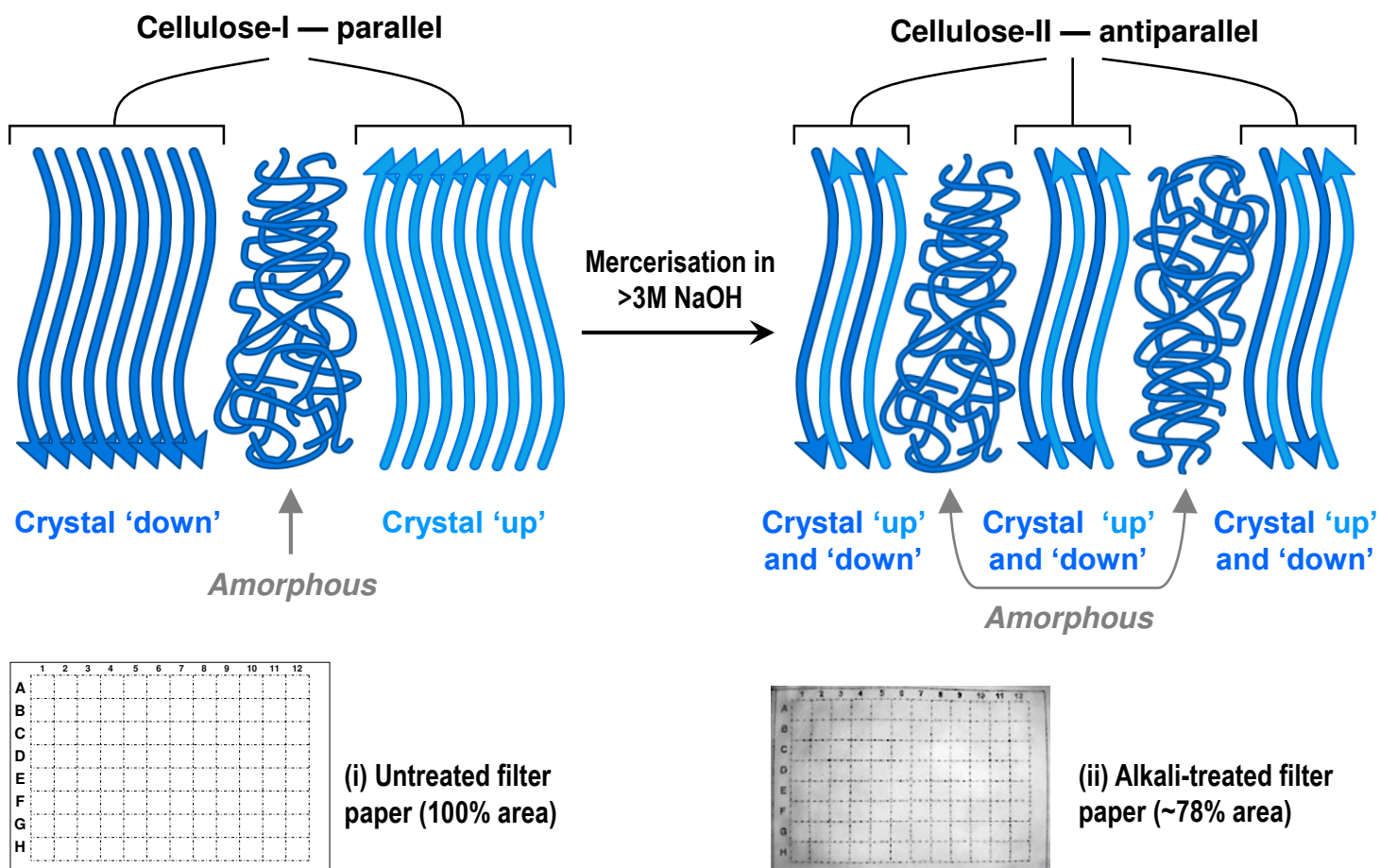


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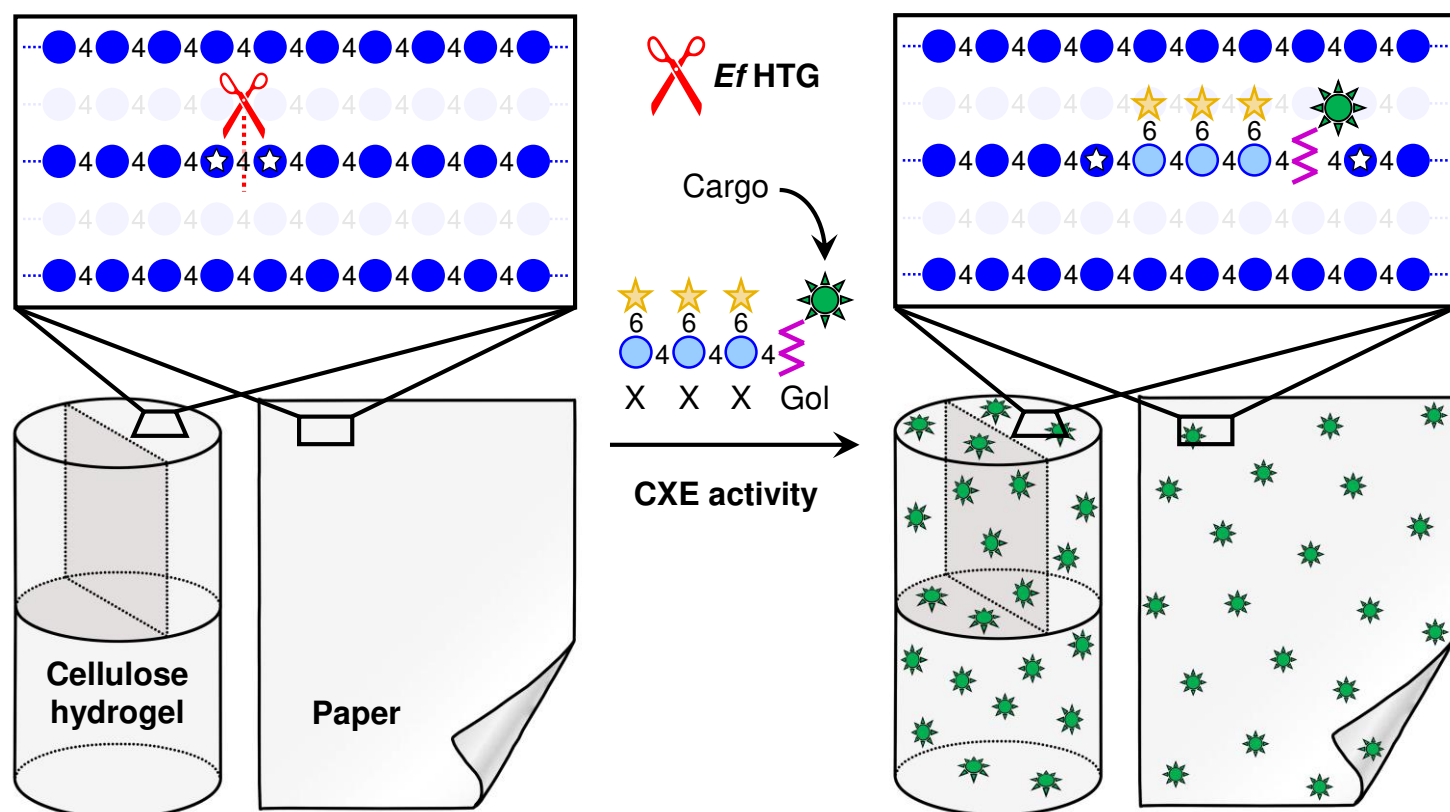


Fig_5

a



b



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4 **polysaccharides via stable covalent bonds**

5

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